Plant Physiol. (1967) 42, 398–406

Gibberellic Acid-Enhanced Synthesis and Release of α-Amylase and Ribonuclease by Isolated Barley Aleurone Layers

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Received December 16, 1966.

Summary. Gibberellic acid enhances the synthesis of α-amylase in isolated aleurone layers of barley-seeds (*Hordeum vulgare* var. Himalaya). In the presence of 20 mM calcium chloride the amount of enzyme obtained from isolated aleurone layers is quantitatively comparable to that of the half-seeds used in earlier studies. After a lag period of 6 to 8 hours enzyme is produced at a linear rate. Gibberellic acid does not merely trigger α-amylase synthesis, but it is continuously required during the period of enzyme formation. Enzyme synthesis is inhibited by inhibitors of protein and RNA synthesis. Small amounts of actinomycin D differentially inhibit enzyme release and enzyme synthesis suggesting 2 distinct processes. Gibberellic acid similarly enhances the formation of ribonuclease which increases linearly over a 48 hour period. During the first 24 hours the enzyme is retained by the aleurone cells and this is followed by a rapid release of ribonuclease during the next 24 hour period. The capacity to release the enzyme is generated between 20 and 28 hours after the addition of the hormone. Ribonuclease formation is inhibited by inhibitors of protein and RNA synthesis. These inhibitors also prevent the formation of the release mechanism if added at the appropriate moment.

Haberlandt observed in 1890 (3) that the aleurone layers of rye produce a substance (or substances) which causes liquefaction of the starchy portion of the endosperm and dissolution of the starch grains. This observation was confirmed by Brown and Escombe (2) who used barley seeds as their experimental material. Many years later Yomo (13) and Paleg (7) showed independently that when gibberellic acid is added to endosperm portions of barley seeds it initates the production of amylolytic enzymes and the release of sugars. Briggs (1) extended this observation to include several other hydrolytic enzymes (protease, phosphatase and β-glucanase).

The only living cells in the endosperm portion of the seed, judged by presence of respiration and amino acid incorporation are those of the aleurone layers and it has been shown that all the α-amylase is produced by the aleurone cells (6, 9). In several varieties of barley it is possible to separate the aleurone cells (with the seed coat) from the starchy portion of the endosperm after the half seeds have been imbibed for 3 days. In this way one can study the effect of the hormone on an isolated tissue only 3 cell layers thick and consisting only of target cells. The effect can be measured in terms of the resulting increase of several hydrolytic enzymes.

In the earlier work of this laboratory (10, 11) isolated aleurone layers were used occasionally for studies with inhibitors. No extensive use was however made of this potentially elegant system because half-seeds and isolated aleurone layers were not quantitatively comparable; the latter produced only a quarter to a third as much α-amylase as the former and we were therefore not sure that they were qualitatively similar either. This problem has now been remedied and we have made a more detailed study of the production and the release of α-amylase and ribonuclease by isolated aleurone layers of barley. Some of the earlier experiments (9, 10) were repeated because we felt it was necessary to define as carefully as possible the isolated aleurone system in order that it may be used to investigate the biochemical mode of action of the hormone. When the experimental results were similar for aleurone layers and half-seeds, they are mentioned in the text, but the results are not given in detail.

Materials and Methods

Seeds of barley (*Hordeum vulgare* var. Himalaya) were cut transversely and the embryo containing halves were discarded. The extreme tips of the distal halves were cut off and the pieces of endosperm obtained in this way (referred to as half seeds) were stored until ready for use. Storage time never exceeded 2 weeks. Half seeds were sterilized for 20 minutes in 1 % sodium hypochlo-

rite (commercial liquid bleach, diluted 1:4), rinsed several times in sterile water and preincubated in petri dishes on moist sterile sand. (Autoclave 10 cm petri dishes containing 100 g of purified sand and moistened with 20 to 21 ml of sterile distilled water). Petri dishes containing 100 to 160 half-seeds were wrapped in aluminum foil and allowed to incubate at room temperature for 3 days. After this time the seed coats with attached aleurone layers were easily removed from the starchy portion of the endosperm with the aid of 2 small spatulas. This dissection was done in a transfer room under aseptic conditions. Ten such aleurone layers were put in a 25 ml Erlenmeyer flask containing the incubation medium. The latter consisted of 2 μmoles of acetate buffer (pH 4.8), 200 μmoles of $CaCl_2$ and 2 mμmoles of gibberellic acid in a final volume of 2.0 ml. These flasks were sterilized by autoclaving. Practical grade gibberellic acid (Sigma Chemical Company) was used throughout the experiments. This preparation contains more than 90 % GA_3 and is referred to in the text as GA. (We did not study the effect of autoclaving on GA, but for experiments involving GA as a critical variable the solutions of gibberellic acid were sterilized by filtration and added to the sterile flasks). In order to insure against any microbial growth, 1 drop (30 μl) of a solution containing 0.5 μg/ml of streptomycin, penicillin and mysteclin or 0.5 mg/ml of chloramphenicol was added to each flask. The aleurone layers were incubated at 25° on a Dubnoff metabolic shaker (40 oscillations per minute). At the end of the incubation period 1 ml of water was added to the flasks and the media were decanted. The aleurone layers were rinsed with 2.5 ml of water and this was combined with the media. The aleurone layers were ground to a thick paste in a porcelain mortar with a little sand and 0.8 ml of 0.2 M sodium chloride. The homogenate was diluted with 4.0 ml of the same solution, poured into a centrifuge tube and centrifuged at 2000 $\times$ g for 10 minutes. The resulting supernatant fraction (referred to as extract) and the medium were assayed for α-amylase activity by the method of Shuster and Gifford (8).

The α-amylase assay was calibrated with purified malt α-amylase and a conversion factor of 2.7 μg of α-amylase per unit of optical density change was used. This conversion factor is dependent on the starch used for the assay. A fresh starch solution was made every day using 150 mg of native (non-solubilized) potato starch (Nutritional Biochemical Corp.), 600 mg of KH_2PO_4, and 200 μmoles of calcium chloride in a final volume of 100 ml. The starch suspension was boiled for 1 minute, cooled and centrifuged at 2000 $\times$ g for 10 minutes. The clear supernatant which was used for the assay was carefully separated from the suspended unsolubilized starch in the bottom of the tube. The α-amylase assay was carried out in a 16 $\times$ 150 mm test tube with a suitable aliquot of enzyme (0.02–0.2 ml) and enough water to make 1.0 ml. The reaction was started by the addition of 1 ml of starch solution, allowed to proceed at room temperature for a suitable time (1–5 min) and stopped by adding 1.0 ml of iodine reagent. The iodine reagent was made by diluting 1.0 ml of iodine stock solution (6 g of KI and 600 mg of iodine dissolved in 100 ml of water) to 100 ml with 0.05 N hydrochloric acid. Five ml of distilled water were added to each tube and after thorough mixing the optical density was read at 620 mμ using a Coleman colorimeter. The initial OD of the starch solution is usually about 1.35. The decrease in OD at 620 mμ caused by the action of the enzyme is proportional to the quantity of α-amylase present and the length of the reaction time over the range of 30 % to 75 % decrease in OD.

Ribonuclease was determined according to the method of Wilson (12). One unit of enzyme activity represents 0.1 OD unit of soluble nucleotides generated during a 40 minute incubation period.

Results

α-Amylase Production by Isolated Aleurone Layers. A comparison of the α-amylase production by half seeds and isolated aleurone layers is shown in table I. After their separation from the starchy portion of the endosperm the aleurone lavers produce at most half as much α-amylase as the intact half seeds, and frequently less than a quarter of the total amount. It was shown earlier (9) that the starch portion of the endosperm does not contribute to the α-amylase production and the discrepancy was therefore ascribed to the existence of another factor (or factors) in the starch endosperm which interacts with the aleurone layers to enhance α-amylase synthesis (11). We observed a small stimulation of α-amylase production when a mixture of 16 amino acids, each at 1 mM, was

Table I. *Production of α-Amylase by Half-seeds and Isolated Aleurone Layers as Affected by GA and $CaCl_2$*

Ten half-seeds or 10 aleurone layers were incubated for 24 hours with buffer, with or without 1 μM GA and with or without 20 mM $CaCl_2$. Activity of the α-amylase was measured in the medium and in an extract of the tissue. The data indicated the sum of these 2 values.

		Total μg of α-amylase produced	
		no $CaCl_2$	20 mM $CaCl_2$
Half-seeds	Control	16	19
	1 μM GA	238	420
Aleurone layers	Control	47	80
	1 μM GA	121	520

Table II. *Effect of Calcium and Other Divalent Ions on the Production of α-Amylase by Aleurone Layers*

Ten half aleurone layers were incubated with buffer and 1 μM GA and the appropriate concentration of the salt. α-Amylase was assayed in the medium and the tissue extract after 24 hours incubation.

Treatment	μg of α-amylase per 10 aleurone layers
Control	69
0.1 mM	74
1 mM	198
10 mM	293
20 mM	328
100 mM	335
10 mM	223
10 mM	42
10 mM	70
10 mM	2

added to the incubation medium of the aleurone layers (J. E. Varner and M. J. Chrispeels, unpublished), but this addition did not bring the production of the aleurone layers up to the level of half seeds. This however, can easily be achieved by the simple expedient of adding 20 mM $CaCl_2$ to the incubation medium. Calcium chloride causes a 2-fold increase in the amount of enzyme obtained from the half seeds and a 4-fold increase when added to aleurone layers. The effect of calcium chloride and chlorides of other divalent ions on aleurone layers is shown in table II. The requirement for calcium is saturated at 20 mM. $SrCl_2$ can be substituted for $CaCl_2$ but $MgCl_2$ and $BaCl_2$ are without effect, while $CdCl_2$ is inhibitory.

Concerning the basis of the calcium effect we first explored the possibility that $CaCl_2$ affects the synthesis of the enzyme, but no evidence to this effect could be obtained. Next, because α-amylase from malt is known to bind calcium we investigated the effect on the activity of the enzyme. Aleurone layers were incubated with various concentrations of calcium for 10 hours and at this time 500 μg of purified malt α-amylase were added. At the end of the 24 hour run the α-amylase activity had almost completely disappeared in the absence of calcium while it remained in the presence of calcium (table III). The data show that the same concentration of $CaCl_2$ which gives maximal activity of the enzyme produced under the influence of GA is necessary for maximal preservation of added α-amylase. Again $SrCl_2$ is a fairly good substitute for $CaCl_2$, while $MgCl_2$ is without effect. If $CaCl_2$ was added to the medium at the end of the experiment (24 hrs) instead of at the beginning, no increase in α-amylase activity was observed. This suggests that we are dealing here with an irreversible inactivation of the enzyme.

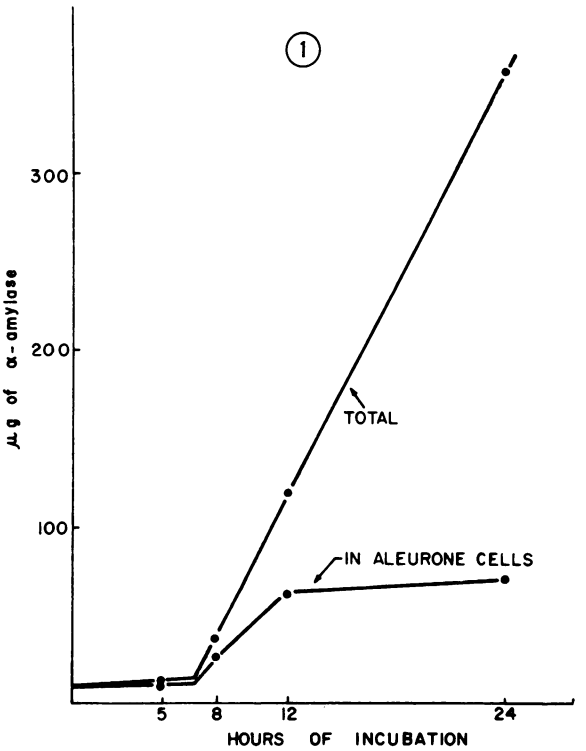

FIG. 1. Timecourse of α-amylase synthesis by 10 aleurone layers incubated with 1 μM GA. Enzyme activity was measured in the medium surrounding the aleurone layers and in the supernatant of a 0.2 M NaCl extract of the aleurone layers. The term total refers to the sum of these 2 activities.

Table III. *Protection of α-Amylase Against Inactivation by $CaCl_2$, $MgCl_2$ and $SrCl_2$*

Ten half aleurone layers were incubated with buffer and 1 μM GA. After 10 hours 500 μg of pure α-amylase isolated from brewers malt were added. After 14 more hours the media and tissue extracts were assayed for α-amylase. Expected production after 24 hours was determined as a separate experiment, without added malt α-amylase. The figures show the amount which was produced in the presence of the same amounts of the different salts.

Treatment	α-Amylase (μg per 10 aleurone layers) Added to the medium after 10 hrs	Assayed after 24 hrs	Expected production after 24 hrs	% Remaining after 24 hrs of the 500 μg added at 10 hrs
Control	500	145	55	18
1 mM $CaCl_2$	500	610	202	82
20 mM $CaCl_2$	500	810	320	98
10 mM $SrCl_2$	500	630	230	80
10 mM $MgCl_2$	500	121	81	8

Table IV. *Effect of GA Concentration on α-Amylase Production*

Ten half-seeds or 10 aleurone layers were incubated for 24 hours with buffer, 20 mM $CaCl_2$ and different concentrations of GA. Activity of the α-amylase was measured in the medium and in an extract of the tissue. The data indicate the sum of these 2 values.

Treatment	μg of α-amylase per 10 aleurone layers
Control	54
0.001 μM GA	157
0.01 μM GA	410
0.1 μM GA	454
1.0 μM GA	451

At the end of the 3 day pre-incubation period the half-seeds contain a small amount of α-amylase, usually less than 1 μg per half aleurone layer. When GA is added the production of the enzyme is linear for 16 to 24 hours after an initial lag-period of 6 to 8 hours (fig 1). Most of the enzyme is secreted into the medium by the aleurone layers, while the cells retain only a small amount which remains constant from 12 to 24 hours. Only a small amount of α-amylase is synthesized in the absence of exogenous GA. These data are similar to those obtained with half-seeds (9, 10, 11).

In some experiments 10 half aleurone layers produce as much as 600 μg α-amylase in 24 hours, in other as little as 275 μg. However, enzyme production is always at a constant linear rate regardless of the amount which is produced in 24 hours. The requirement for GA is satisfied at a concentration between 0.01 μM and 0.1 μM (table IV).

Figure 2 shows that there is a continuous requirement for GA during the period of enzyme synthesis and that it is not sufficient to have GA present only during the induction period. Aleurone layers were rinsed, to remove the GA, 11 hours after the start of the incubation. After the removal of GA α-amylase synthesis continued at a slightly reduced rate for approximately 6 hours. During the next 6-hour period it continued at 50 % of the rate present in the presence of GA. That substantial enzyme synthesis continues after removal of GA may be due to GA remaining in the tissue.

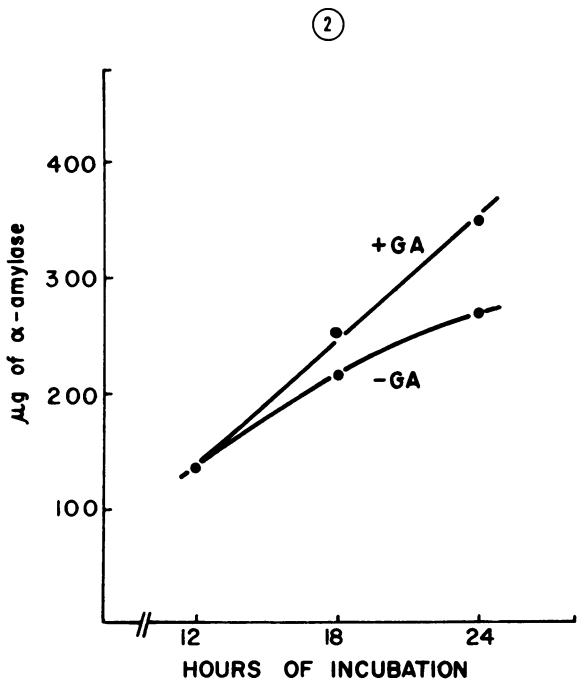

FIG. 2. Effect of the removal of GA after α-amylase synthesis has been initiated. Aleurone layers were incubated in 10 mμM GA for 11 hours. GA was then removed by frequent rinsing and at 12 hours GA was either added or withheld subsequently.

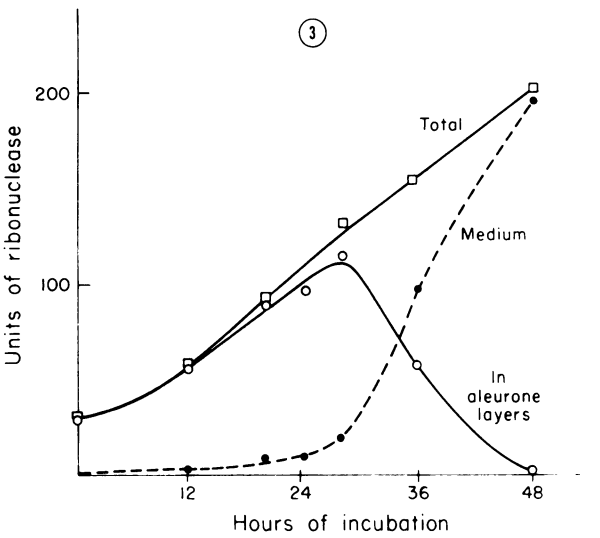

FIG. 3. Time course of production of ribonuclease by 10 aleurone layers incubated with 5 mμM GA.

Ribonuclease Production by Isolated Aleurone Layers. Ribonuclease is synthesized by the aleurone layers in response to added GA at a linear rate, which continues for at least 48 hours after the start of the experiment (fig 3). Almost no ribonuclease is released into the medium in the first 24 hours, causing it to accumulate in the aleurone cells. This is followed by a rapid release of the accumulated enzyme in the next 24 hours, and 48 hours after the start of the incubation no ribonuclease remains in the aleurone layers. This suggests that the aleurone layers do not have the capacity to secrete the enzyme until 24 to 28 hours after the addition of GA.

Isolated aleurone layers produce as much ribonuclease as half-seeds indicating that the aleurone cells are the source of the enzyme. Dry half-seeds contain a small amount of ribonuclease (about 15 units per 10 half-seeds). In 1 experiment seeds

Table V. *Increase in Ribonuclease during Pre-incubation of Half-seeds*

Sterile half-seeds were incubated on moist sterile sand for the number of days indicated in the left column. At the end of the incubation they were homogenized in 0.2 N NaCl and ribonuclease was assayed in this extract of the tissue.

Days of pre-incubation	Units of ribonuclease per 10 half-seeds
0 (Dry half-seeds)	14.2
1	17.1
2	23.2
3	33.8
4	45.1
3 With 10 μg/ml cycloheximide	17.4

were handled with gloves to make sure that no enzyme was transferred from the hands to the seeds. There is a steady rise in the endogenous level of ribonuclease during pre-incubation of the half-seeds on moist sand (table V). This ribonuclease is also located in the aleurone cells and not in the starchy portion of the half-seed endosperm (unpublished results).

The requirement for GA is satisfied at a concentration of 1 mμM (fig 4), which is well below that required for maximal α-amylase synthesis. Ribonuclease synthesis also occurs in the absence of GA and the addition of GA causes only a 2-fold increase in the amount of ribonuclease produced in 48 hours. Most of the ribonuclease synthesized in the absence of GA and in the presence of low concentrations of GA (0.01 and 0.1 mμM) remains in the aleurone layers after 48 hours. The rapid rise in the amount of ribonuclease released into the medium over the concentration range 0.3 to 3 mμM suggests that both synthesis and release of the enzyme are independently controlled by GA.

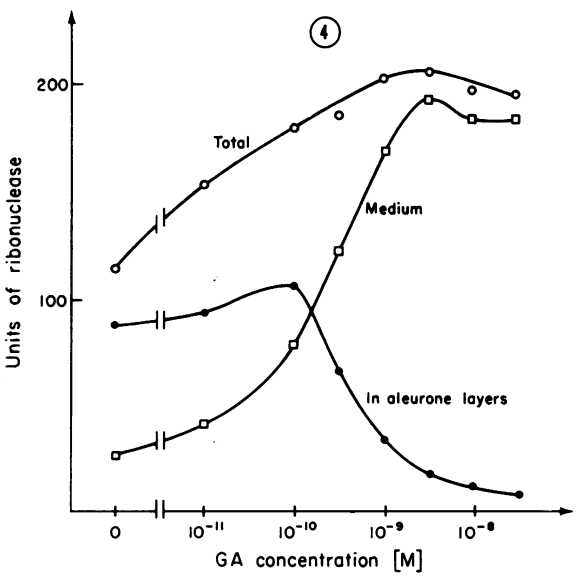

FIG. 4. Dose response curve of the production of ribonuclease by aleurone layers at different concentrations of GA. Ribonuclease was measured in the medium and an extract of the tissue after 48 hours of incubation.

Effect of Metabolic Inhibitors on α-Amylase Synthesis. The production of α-amylase by aleurone layers is inhibited by the uncouplers 2,4-dinitrophenol and carbonylcyanide-*m*-chlorophenyl hydrazone (CCP) and by the protein synthesis inhibitors cycloheximide, puromycin, and *p*-fluorophenylalanine. These data are not reported here because they are similar to those obtained with half-seeds (9, 10, 11). The formation of the enzyme can also be arrested by the addition of the inhibitors in mid-course during enzyme production (e.g. 12 or 15 hrs after the addition of GA). Table VI shows the effect of cycloheximide and CCP added 15 hours after GA. Both inhibitors stop α-amylase synthesis immediately. This eliminates the possibility that any part of the α-amylase exists as an enzymatically inactive precursor which is being converted into an active enzyme under the influence of GA.

Table VI. *Mid-course Inhibition of α-Amylase Formation by Cycloheximide and CCP*

Ten aleurone layers were incubated with buffer, 1 μM GA and 20 mM $CaCl_2$. Activity of the α-amylase was measured in the medium and in an extract of the tissue. The data indicate the sum of these 2 values. Inhibitors (CH = cycloheximide 2 μg/ml; CCP = carbonylcyanide-*m*-chlorophenylhydrazone 20 μM) are added at 15 hours and the samples are assayed 4 hours later.

Treatment	μg of α-amylase per 10 aleurone layers
GA for 15 hrs	196
GA for 19 hrs	370
Add CH at 15 hrs	220
Add CCP at 15 hrs	189

Synthesis of α-amylase is inhibited by actinomycin D, an inhibitor of RNA synthesis, and by some base analogues (8-azaguanine and 5-azacytidine) but not by others (5-bromouracil and 5-fluorouracil). When the antibiotic is added at the same time as GA a high concentration is needed to prevent α-amylase formation (table VII). Even 100 μg/ml of actinomycin D inhibits α-amylase synthesis by only 60 %. In a parallel experiment we measured the inhibition of ^{14}C-uridine incorporation by actinomycin D in the 4 to 8 hour period after the addition of the antibiotic. In this experiment no GA was added to avoid the complicating factor of a possible increase in the pool size of uridine due to the presence of GA-enhanced ribonuclease. The inhibitory action of actinomycin D on the incorporation of ^{14}C-uridine increases with

Table VII. *Inhibition of α-Amylase Production by Actinomycin D*

Samples of 10 aleurone layers were incubated with buffer, 1 μM GA and 20 mM $CaCl_2$. Appropriate amounts of actinomycin D were added at the same time as GA. The enzyme was assayed after 24 hours incubation. In a parallel experiment aleurone layers were incubated without GA and allowed to incorporate ^{14}C-uridine (1 μc per flask specific activity 26 mc per μmole) between 4 and 8 hours after the addition of actinomycin D. RNA was extracted by the method of Kirby (5).

Treatment	α-Amylase μg Per 10 aleurone layers	α-Amylase % Inhibition	% Inhibition of incorporation of ^{14}C-uridine
Control	359	...	...
20 μg/ml Act. D	332	7 %	40 %
50 μg/ml Act. D	241	33 %	63 %
100 μg ml Act. D	152	58 %	75 %

increasing concentrations of the antibiotic. The lowest concentration of actinomycin D (25 μg/ml) which hardly affects α-amylase synthesis inhibits uridine incorporation by only 40 %. The data indicate that the failure of actinomycin D, especially at lower concentrations, to inhibit α-amylase synthesis may be related to its ineffectiveness in blocking RNA synthesis in this system.

The lower concentrations of actinomycin D which have relatively little influence on enzyme formation have a profound effect on the release of α-amylase by the aleurone cells (table VIII). In the presence of 25 μg/ml of actinomycin D more than half the α-amylase is retained by the aleurone cells, instead of the usual 15 to 20 %. Enzyme release is proportionally more affected than enzyme synthesis indicating that the process may be affected independently.

Table VIII. *Inhibition of α-Amylase Secretion by Actinomycin D*

Details the same as in table VII.

Treatment	μg Of α-amylase per 10 aleurone layers		
	Medium	Extract	Total
Control	324	79	403
25 μg/ml of Act. D	176	197	373
50 μg/ml of Act. D	108	194	302

Effect of Metabolic Inhibitors on Ribonuclease Production and Release. Inhibitors of protein synthesis inhibit the formation of ribonuclease by aleurone layers. Cycloheximide (5 μg/ml), *p*-fluorophenylalanine (2.5 mM) and puromycin (250 μg/ml) inhibit enzyme synthesis by 98 %, 52 % and 22 % respectively. The increase in ribonuclease which is observed during the 3 day pre-incubation period is also inhibited by cycloheximide (table V). When the inhibitor is added in midcourse (e.g. 24 hrs after GA) it inhibits ribonuclease formation very rapidly, without inhibiting the release of the enzyme from the aleurone layers

Table IX. *Cycloheximide Inhibits Ribonuclease Synthesis when Added 20 Hours After GA*

Ten aleurone layers were incubated with buffer, 20 mM $CaCl_2$ and 20 mμM GA. After 24 hours 5 μg/ml of cycloheximide was added to 1 set.

	Units of RNAase per 10 aleurone layers		
	Medium	Extract	Total
0 Hrs	...	28.0	28.0
24 Hrs	8.5	98.1	106.6
48 Hrs	219.5	11.5	231.0
48 Hrs, add cycloheximide at 24 hrs	100.6	17.4	118.0

in the following 24 hour period (table IX). This indicates that cycloheximide does not inhibit the release process per se.

An experiment was designed to test the possibility that the formation of the release capacity requires protein synthesis. Time course studies on the appearance of ribonuclease in the medium had shown that this capacity develops between 20 and 28 hours after the addition of GA (fig 3). Cycloheximide was added to the aleurone layers 20, 24 and 28 hours after GA and the release of ribonuclease from the aleurone layers was measured in 2 4-hour periods following the addition of cycloheximide (table X). In this way it is possible to study enzyme release in the absence of enzyme synthesis.

The results show that if cycloheximide is added 20 hours after GA, release of the enzyme is slow, even between 24 and 28 hours, indicating that the release capacity is not developing in these aleurone layers. The enzyme is released more rapidly if the inhibitor is added after 24 or 28 hours. Most or all of the release capacity has already developed at this stage.

Ribonuclease synthesis is also inhibited by actinomycin D (50 and 100 μg/ml). A time course study indicates that there is no inhibition in the first 24 hours, while the inhibition is almost complete in the next 24 hours (table XI). Actinomycin

Table X. *Release of Ribonuclease from Aleurone Layers after Inhibition with Cycloheximide*

Aleurone layers were incubated for 20, 24 or 28 hours with 5 mμM GA. At these times they were rinsed 3 times for 5 minutes with sterile medium containing GA and 10 μg/ml of cycloheximide to remove the ribonuclease which had already been released in the medium. The aleurone layers were then further incubated with medium containing 5 mμM of GA and 10 μg/ml of cycloheximide for 4 and 8 hours. The aleurone layers were collected and the ribonuclease remaining in the aleurone cells was determined. Units of ribonuclease released were calculated by difference.

Time or addition of cycloheximide	Time period after cycloheximide	Units of ribonuclease per 10 aleurone layers	Units of ribonuclease released in 4 hrs
20 hrs	Initial	75.6	
	After 4 hrs	59.1	16.5
	After 8 hrs	47.7	11.4
24 hrs	Initial	107.0	
	After 4 hrs	59.3	47.7
	After 8 hrs	37.4	21.9
28 hrs	Initial	109.1	
	After 4 hrs	35.7	73.4
	After 8 hrs	17.8	17.9

Table XI. *Inhibition of Ribonuclease Synthesis and Secretion by Actinomycin D*

Aleurone layers were incubated in the presence of 5 mμM GA and actinomycin D.

Treatment	Time	Units of ribonuclease per 10 aleurone layers		
		Medium	Extract	Total
Control	24 hrs	8.7	94.2	106.9
Act. D 50 μg/ml		9.3	82.4	91.7
Act. D 100 μg/ml		5.7	86.7	92.4
Control	48 hrs	191.5	3.7	195.2
Act. D 50 μg/ml		86.0	24.4	110.4
Act. D 100 μg/ml		18.2	78.9	97.1

Table XII. *Inhibition of Ribonuclease Release by Actinomycin D*

Details as in table X. To 1 set actinomycin D (100 μg/ml) was added from the beginning. After 26 hours the aleurone layers were rinsed and further incubated in a similar medium containing 10 μg/ml of cycloheximide. The ribonuclease remaining in the aleurone layers was measured 4 and 8 hours later.

	Time period after cycloheximide	Units of ribonuclease per 10 aleurone layers	Units of ribonuclease released in 4 hrs
Actinomycin D	Initial	72.6	...
	After 4 hrs	73.2	0
	After 8 hrs	62.0	9.4
Control	Initial	70.6	...
	After 4 hrs	47.6	23.0
	After 8 hrs	22.8	24.6

D also prevents the release of the enzyme from the aleurone layers in the second 24 hour period, suggesting that RNA synthesis may be required for the formation of the release capacity.

The effect of actinomycin D on the development of the release capacity was measured in the following, more direct way. Aleurone layers were incubated for 26 hours with GA and 100 μg/ml of actinomycin D and then treated with cycloheximide to stop any further ribonuclease synthesis. By this time (26 hrs) most of the release capacity will have developed in the controls and cycloheximide will not have a profound influence on the release of the enzyme (see data in table X). This allows us to measure the effect of actinomycin D on the development of the release capacity in the absence of further ribonuclease synthesis and in the absence of the effect which cycloheximide itself has on the development of the release capacity. Release of enzyme into the medium was then measured over the next 2 4-hour periods. The data in table XII show that release is almost completely inhibited if the aleurone layers have been incubated with actinomycin D for 26 hours. Addition of actinomycin D to the controls, 26 hours after GA (the beginning of the actual release experiment) had no effect on

the rate of release indicating that the antibiotic does not interfere with the release process itself.

Discussion

Using isolated aleurone layers we have confirmed our earlier results on gibberellic acid induced α-amylase synthesis obtained with half-seeds. Previously work carried out with isolated aleurone layers (9, 10, 11) was hampered by the fact that they seemed able to produce only smaller amounts of α-amylase than half-seeds. This problem has been remedied by showing that calcium ions are needed for obtaining maximal enzyme activity and we can now be assured that aleurone layers are comparable to half-seeds quantitatively as well as qualitatively. This is also true for the GA-triggered synthesis of ribonuclease.

One problem remains: detaching the aleurone layers from the starchy endosperm initiates the formation of a small amount of α-amylase and a large amount of ribonuclease and increases the background against which one has to work. It is noteworthy that the enzymes have different optima in their GA requirement. A concentration of 2 mμM GA is enough to obtain maximal ribonuclease synthesis, but this constitutes only a 2-fold increase over the control value. Is it possible that enzyme synthesis in the absence of added GA is due to endogenous gibberellins? To test this possibility 350 aleurone layers were extracted according to the method of Kende and Lang (4), and the purified extract was bioassayed with the d_5-dwarf corn test. The results indicated that aleurone layers do contain gibberellin-like substances (Chrispeels and Jones, unpublished data). Whether these endogenous gibberellin-like substances can trigger off the synthesis of α-amylase or ribonuclease remains unknown. It is, however, conceivable that processes which are triggered off by small amounts of GA (e.g. ribonuclease synthesis) can be initiated more easily without added GA than processes which require larger amounts of hormone (e.g. α-amylase synthesis). This could account for the observation that in the absence of GA relatively more ribonuclease is synthesized than α-amylase.

Enzyme synthesis can be blocked by the addition of uncouplers of phosphorylation or inhibitors of protein synthesis added either at the same time as GA or in mid-course when enzyme synthesis is in progress. Both kinds of inhibitors stop enzyme synthesis almost immediately. This eliminates the possibility that there is an enzymatically inactive precursor which is converted into active enzymes, unless it is assumed that this conversion is in turn dependent on continuous protein synthesis, because it is brought about by an unstable enzyme. Enzyme synthesis is also blocked by inhibitors of RNA synthesis suggesting that a new RNA is required for the synthesis of the enzymes.

It is usually difficult to separate experimentally the release of an enzyme from its synthesis. Aleurone layers contain only a small amount of α-amylase and this enzyme is therefore not suitable for a study of release. Time curves of enzyme synthesis indicate that α-amylase is released as soon or almost as soon as it is synthesized. The only way in which the 2 processes could be separated is by inhibition with actinomycin D. Small amounts of this antibiotic (25 μg/ml) inhibit α-amylase release much more severely than its synthesis. A different picture is obtained with ribonuclease. No ribonuclease is released in the first 24 hours and in the absence of GA or in the presence of small amounts of GA very little enzyme is released in the next 24 hours. Larger amounts of GA on the other hand trigger a very rapid release of the enzyme in the second 24 hour period. The release capacity develops between 20 and 28 hours after the addition of GA. Addition of cycloheximide after 20 hours, but not after 28 hours, stops the development of the release capacity. Addition of actinomycin D at the same time as GA does not inhibit enzyme synthesis during the first 24 hours, but inhibits both synthesis and release in the next 24 hour period. It appears therefore that enzyme synthesis and release are activated and inhibited differentially, indicating that they are 2 distinct processes. However, they may be interdependent and the possibility that an inhibition of release results in an inhibition of synthesis cannot be excluded.

Whether we are dealing with an active secretion or with a possible release of the enzymes due to necrosis of the cells is not yet clear. After 24 hours the cells have lost a large portion of their nitrogen (11) and visual observation (Varner, unpublished) shows them to be completely vacuolated. However, respiration remains unchanged for 24 hours, then declines slowly whether GA has been added or not. This indicates that the biochemical machinery of the cells remains intact, at least during the first 24 hours, and there is no reason why the aleurone cells should not be able to carry out a controlled release. The release of α-amylase follows a different pattern from that of ribonuclease suggesting again a specific mechanism.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Miss Nancy Joseph. We wish to thank R. A. Nilan for supplying the Himalaya variety of barley. The actinomycin D was a gift from Merck, Inc. This work was supported by the United States Atomic Energy Commission under contract No. At (11-1) 1338.

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